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TO: Assistant Commissioner for Patents
Box Patent Applications
Washington D.C. 20231

Attorney Docket No. 026083/0119
(must include alphanumeric codes if no inventors named)

UTILITY PATENT APPLICATION TRANSMITTAL
(new nonprovisional applications under 37 CFR 1.53(b))

Transmitted herewith for filing is the patent application of:

INVENTOR(S): Klaus BOSSLET, Jorg CZECH and Dieter HOFFMAN

**TITLE: BIFUNCTIONAL GLYCOPROTEINS HAVING A MODIFIED CARBOHYDRATE
COMPLEMENT, AND THEIR USE IN TUMOR-SELECTIVE THERAPY**

In connection with this application, the following are enclosed:

APPLICATION ELEMENTS:

XX Specification - 40 TOTAL PAGES

(preferred arrangement:)

- Descriptive Title of the Invention
- Cross Reference to Related Applications
- Statement Regard Fed sponsored R&D
- Reference to Microfiche Appendix
- Background of the Invention
- Brief Summary of the Invention
- Brief Description of the Drawings (if filed)
- Detailed Description
- Claim(s)
- Abstract of the Disclosure

XX Drawings - Total Sheets 8

XX Declaration and Power of Attorney - Total Sheets 2

 Newly executed (original or copy)

XX Copy from a prior application (37 CFR 1.63(d))

(relates to continuation/divisional boxes completed) - NOTE: Box below

 DELETION OF INVENTOR(S) - Signed statement attached deleting inventor(s)
named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).

XX **Incorporation By Reference** (useable if copy of prior application Declaration being submitted)

The entire disclosure of the prior application, from which a COPY of the oath or declaration is supplied as noted above, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

 Microfiche Computer Program (Appendix)



Utility Patent Application Transmittal Page 2

Attorney Docket No. 026083/0119 - Foley & Lardner

XX Nucleotide and/or Amino Acid Sequence Submission (if applicable, all necessary)

 Computer Readable Copy

XX Paper Copy (identical to computer copy)

XX Statement verifying identify of above copies

ACCOMPANYING APPLICATION PARTS

 Assignment Papers (cover sheet & document(s))

 37 CFR 3.73(b) Statement (when there is an assignee)

 English Translation Document (if applicable)

XX Information Disclosure Statement(IDS) with PTO-1449. Copies of IDS Citations

XX Preliminary Amendment

XX Return Receipt Postcard (MPEP 503)

 Small Entity Statement(s)

 Statement file in prior application, status still proper and desired.

 Certified Copy of Priority Document(s) with Claim of Priority
(if foreign priority is claimed).

 OTHER:

If a **CONTINUING APPLICATION**, check appropriate box and supply the requisite information:

XX Continuation Divisional Continuation-in-part (CIP)

of prior application Serial No. 08/663,406.

Amend the specification by inserting before the first line the following sentence: --This application is a XX continuation, divisional or continuation-in-part of application Serial No. 08/663,406, filed June 13, 1996; which is a continuation of 08/235,395 filed April 29, 1994.--

CORRESPONDENCE ADDRESS:

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FEE CALCULATIONS: (Small entity fees indicated in parentheses.)

(1) For	(2) Number Filed	(3) Number Extra	(4) Rate	(5) Basic Fee \$760 (\$380)
Total Claims	21 - 20 =	1	x \$18 (x \$9)	18.00
Independent Claims	1 - 3 =	0	x \$78 (x \$39)	0.00
Multiple Dependent Claims			\$260 (\$130)	0.00
Assignment Recording Fee per property			\$40	0.00
Surcharge Under 37 C.F.R. 1.16(e)			\$130 (\$65)	0.00
TOTAL FEE:				\$778.00

METHOD OF PAYMENT:

XX A check in the amount of the above TOTAL FEE is attached. If payment enclosed, this amount is believed to be correct; however, the Commissioner is hereby authorized to charge any deficiency or credit any overpayment to Deposit Account No. 19-0741.

___ If payment by check is NOT enclosed, it is requested that the Patent and Trademark Office advise the undersigned of the period of time within which to file the TOTAL FEE.

Respectfully submitted,

Date: April 30, 1999
Docket No: 026083/0119

Patricia H. Sandercock 33,683
for Colin G. Sandercock
Registration No. 31,298

002.211850

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Docket No. 026083/0119

In re patent application of

Klaus BOSSLET, *et al.*

Group Art Unit: Unknown

Serial No. Cont. of 08/663,406

Examiner: Unknown

Filed: April 30, 1999

For: BIFUNCTIONAL GLYCOPROTEINS HAVING A MODIFIED
CARBOHYDRATE COMPLEMENT, AND THEIR USE IN TUMOR-
SELECTIVE THERAPY

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to examination of the above-identified application, Applicants respectfully
request the amendment of the application as follows:

IN THE SPECIFICATION

Page 35, at the end of the specification, before the claims, insert the printed
Sequence Listing submitted concurrently herewith, and renumber pages 1-3 of the
Sequence Listing as pages 36-38 of the specification.

IN THE CLAIMS:

Please renumber pages 36-39 as pages 39-42.

REMARKS

Applicants submit this Preliminary Amendment to indicate the insertion point for
the Sequence Listing filed concurrently herewith and to effect the necessary changes in
pagination. Applicants respectfully request examination on the merits of this application.

Applicants herewith submit a Statement Regarding Submission of Sequence Listing instructing the STIC personnel to utilize the computer readable disk filed in the parent application, U.S. Serial No. 08/663,406 on August 29, 1996. Also attached is a paper copy of the Sequence Listing from the parent application. The Sequence Listing filed in U.S. Serial No. 08/663,406 and the present application are identical.

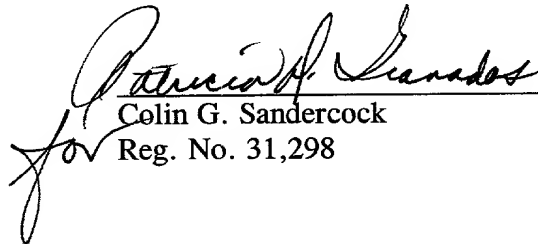
It is believed that no additional fees are required; however, the Commissioner is hereby authorized to charge any deficiency or credit any overpayment to Deposit Account No. 19-0741.

It is further believed that no additional petition for an extension of time under 37 C.F.R. § 1.136 is required. However, should such a petition be required, applicant hereby petitions the Commissioner for an extension of time, and authorizes the Commissioner to charge the necessary petition fee to Deposit Account No. 19-0741.

Respectfully submitted,

April 30, 1999
Date

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BIFUNCTIONAL GLYCOPROTEINS HAVING
A MODIFIED CARBOHYDRATE COMPLEMENT, AND
THEIR USE IN TUMOR-SELECTIVE THERAPY

Background of the Invention

5 Field of the Invention

 The invention relates to bifunctional glycoproteins having targeting protein and enzyme properties. More particularly, the invention relates to such proteins whose complements of carbohydrate residues have been
10 modified in a manner that enhances the clearance of such proteins from the circulation and increases the relative binding of the proteins at the tumor site. The enzymatic portion is capable of converting a prodrug into a cytotoxic drug that attacks tumor cells.

15 This invention also relates to the treatment of tumors with such proteins, and the production of such proteins, including recombinant production and production by transgenic animals.

20 Description of the Background Art

 In efforts to control tumors, attempts have been made in the last twenty years to achieve selective therapeutic effects based on the specificity of antibodies. However, important therapeutic successes still have not been
25 achieved in the case of solid tumors. Although highly specific tumor-selective monoclonal antibodies are available for targeting purposes, the lack of success in immunotherapy is primarily due to the small quantities of monoclonal antibody molecules that can be localized to
30 solid tumors. One reason for this low degree of localization, which is generally insufficient for therapeutic purposes, is the presence of diffusion barriers in the tumor (Jain, R.K., *Cancer Res.* 47: 3039 (1987)). Prior attempts to compensate by increasing the
35 dosage of the drug have encountered problems of widespread non-specific binding in non-tumor structures, and generalized toxic side effects.

 Prior art compounds have sought to utilize (i) the specificity of a monoclonal antibody or tumor-binding

protein partner and (ii) the catalytic amplification potential of an enzyme. Such antibody-enzyme conjugates can be administered to a patient and given time to bind to the tumor. Thereafter, a non-toxic prodrug, which can be cleaved by the enzyme portion of the conjugate to yield a cytotoxic drug, is administered to the patient. In theory, the enzyme portion of the molecules bound to the tumor converts the prodrug in the vicinity of the tumor into a drug which is cytotoxic to the tumor. In reality, however, such compounds suffer several drawbacks.

First, such antibody-enzyme conjugates are highly immunogenic in humans, since they represent chemical conjugates composed, as a rule, of mouse antibodies and xenogeneic enzymes. Repeated use of the same antibody-enzyme conjugate on the same patient is therefore not possible clinically (Bagshawe et al., *Disease Markers*, 9: 233 (1991)).

Second, the conjugates are only relatively slowly removed from the plasma, so that selective and effective prodrug activation is only possible if the elimination of the unwanted non-bound enzyme activity from the plasma is significantly enhanced.

The above-mentioned problem of the immunogenicity of xenogeneic antibody-enzyme conjugates is largely solved by using a recombinant fusion protein that is composed of purely human components. Details for the production of such fusion proteins are described in European Patent Application EP-A-0 501 215, which is incorporated by reference to the extent that it discloses such fusion proteins. In that publication, proteins are described, for example, of the general formula hutuMab-L- β -gluc, with hutuMab being a humanized, or human, tumor-specific monoclonal antibody, or a part thereof which still binds to the tumor, L representing a linker moiety, and β -gluc denoting human β -glucuronidase.

However, in carrying out pharmacological tests on such a fusion protein, it was unexpectedly found that,

even at very short periods of time (1 -3 minutes) after i.v. injection of the fusion protein into human tumor-carrying nude mice, significant quantities of the protein were bound to tumor cells in regions which are close to the blood vessels (easily accessible sites = EAS). Further, at these early times, large quantities of the fusion protein were still present in the plasma, so that selective and effective activation of a suitable prodrug in the tumor was not possible at this early time point after injection.

One proposed solution to the above problem is described in the International Patent Application WO 89/10140, which discloses a three component system for treatment of malignant diseases. The first component localizes at the tumor and has enzymatic activity, e.g., an antibody-enzyme conjugate. The second component is able to bind to the first component and inactivate the catalytic site and/or accelerate the clearance of the first component from the plasma. The third component is a prodrug which can be converted by the enzymatic activity of the first component to form the cytotoxic substance used to treat the tumor.

The three components of 89/10140 are designed to be administered sequentially, not simultaneously. That is, the first component is administered and given sufficient time to localize at the tumor site. The second component is then administered after the first component has localized. Later, the third component is given.

The process of WO 89/10140 has drawbacks, however. First, it has the disadvantage of being a more complex process than the two step processes of the prior art. Moreover, by introducing an additional substance to the human or animal body, particularly the second compound, the risk of side effects and/or adverse reactions, such as the development of an unwanted immune reaction, is increased.

Accordingly, there remains a need for improved compounds and methods for selectively targeting tumors with cytotoxic drugs.

SUMMARY OF THE INVENTION

5 The present inventors have developed solutions to the foregoing problems that make it possible to achieve the desired therapeutic effects using a simpler, two step approach. In their investigation the present inventors prepared bifunctional compounds having both a tumor
10 binding moiety and a catalytic moiety, which compounds are cleared very rapidly from the plasma. In doing so, it was expected that the incidence of binding to the tumor would be decreased due to the short time the compounds were in the plasma. Surprisingly, however,
15 despite the very short presence of the compounds in the plasma, the relative binding of the compounds to the tumor increased. Moreover, to achieve the enhanced clearance, no additional compounds were administered either simultaneously with or subsequently to
20 administration of the bifunctional compounds.

Thus, one aspect of the invention involves, in a first treatment step, administering intravenously ("i.v.") to tumor patients a compound comprising a
bifunctional glycoprotein or bifunctional glycoprotein
25 conjugate, the compound comprising a first portion that possesses an enzyme activity and a second portion that preferentially binds to a tumor-specific antigen. The carbohydrate complement of the compound comprises at least one exposed carbohydrate residue selected from the
30 group consisting of mannose, galactose, N-acetylglucosamine, N-acetyllactose, glucose and fucose, which exposed residue is responsible for the advantageous binding and clearance characteristics of the compound. The enzyme activity of the first portion cleaves a non-
35 toxic drug, which is administered to the subject either concurrently with or subsequently to the administration

of the compound, to a form that is cytotoxic to the tumor cells.

For convenience, the term modified carbohydrate complement or the like will be used herein to denote a carbohydrate complement of the glycoprotein that comprises at least one exposed carbohydrate residue selected from the group consisting of mannose, galactose, N-acetylglucoseamine, N-acetylactose, glucose and fucose.

Thus, in one aspect of the invention there is provided a bifunctional fusion glycoprotein ("FUP") containing a tumor targeting portion, an enzyme portion, and a modified carbohydrate complement. The modified carbohydrate complement contributes to an increased relative concentration of the FUP bound to a tumor and an enhanced clearance of the FUP from the general circulation and non-specific binding sites. The enzyme portion of the FUP is capable of cleaving a non-toxic drug into a tumor cytotoxic drug.

In another aspect of the invention, methods are provided for producing the FUPs having modified carbohydrate complements by colony selection, recombinant DNA and transgenic animal techniques, and chemical or enzymatic reactions.

In yet another aspect of the invention, a bifunctional antibody-enzyme conjugate ("AEC") having a modified carbohydrate complement is provided, wherein the antibody moiety is directed to an epitope on a tumor-specific antigen, and the enzyme is capable of converting a non-toxic drug into a tumor cytotoxic drug.

In still another aspect of the invention there are provided methods for appropriately modifying the carbohydrate complement of an AEC.

These and other aspects of the invention will become readily apparent by reference to the description of the invention and appended claims.

DETAILED DESCRIPTION OF THE FIGURES

Fig. 1 shows the amplification of the V_H and V_L genes. The V_H gene, including its own signal sequence, is amplified (Güssow et al., *Meth. Enzymology*, 203: 99 (1991)) from pABstop 431/26 hum V_H using the oligonucleotides pAB-Back and Linker-Anti (Table 1). The V_L gene is amplified from pABstop 431/26 hum V_L using the oligonucleotides Linker-Sense and $V_{L(Mut)}$ -For (Table 2).

Fig. 2 shows a PCR fragment composed of the V_H that is connected to the V_L gene via a linker.

Fig. 3a shows the removal of the *Hind* III to *Bgl*II restriction fragment from the plasmid pAB 431 VH to produce a vector.

Fig. 3b shows the insertion of the PCR fragment from Fig. 2 into the vector from Fig. 3A to produce the plasmid pMCG-E1, which clone contains the humanized sFv 431/26, a hinge exon, and the complete β -glucuronidase, which clone is transfected into BHK cells.

Fig. 4 shows the plasmid pRMH 140 that carries a neomycin resistance gene into transfected BHK cells.

Fig. 5 shows the plasmid pSV2 that carries the methotrexate resistance gene into transfected BHK cells.

Fig. 6 shows the PCR amplification scheme. The sFv 431/26 fragment (a) is employed as the template for a PCR using the oligos pAB-Back (Table 2) and sFv-For (Table 5). This results in *Bg*III and *Hind*III cleavage sites being introduced at the 3' end of the newly generated sFv 431/26 fragment (b). The PCR fragment is purified and digested with *Hind*III, and then ligated into a pUC18 vector which has been cut with *Hind*III and treated with alkaline phosphatase. The plasmid clone pKB01 is isolated, containing the sFv fragment with the *Bg*III cleavage site.

Fig. 7 shows the amplification of the gene encoding the *E. coli* β -glucuronidase from the vector pRAJ275 by PCR using the oligos *E. coli* β -gluc-Back1 (Table 6) and *E. coli* β -gluc.-For (Table 7), and at the same time provided with a *Bg*III cleavage site, an *Xba*I cleavage

site and, at the 5' end, with a sequence encoding a linker. The resulting fragment is purified and digested with *Bg*III/*Xba*I, and then cloned into the vector pKB01, which has likewise been digested with *Bg*III/*Xba*I. The
5 plasmid clone pKB02 is isolated, containing sFv 431/26 linked to the *E. coli* β -glucuronidase via a linker sequence.

Fig. 8 shows the sFv-*E. coli* β -gluc. fragment, obtained from vector pKB02 by digesting with
10 *Hind*III/*Xba*I, is purified and then ligated into the expression vector pABstop, which has also been cut with *Hind*III/*Xba*I. The plasmid clone pKB03 is isolated, containing the humanized sFv 431/26, a linker and the complete *E. coli* β -glucuronidase.

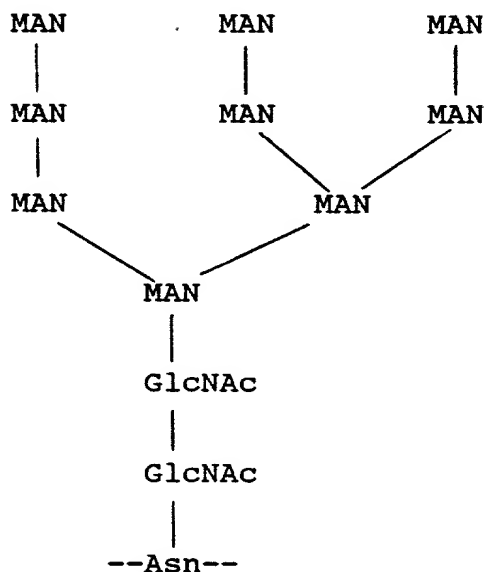
15 DETAILED DESCRIPTION OF THE INVENTION

It has been discovered that solid tumors in a subject may be treated efficiently *in vivo* with cytotoxic drugs, with no or lessened deleterious effect of the cytotoxic drugs on non-tumor tissues, by administering a
20 carbohydrate complement-modified FUP or AEC of this invention with a prodrug. The targeting portion of the FUP or the targeting antibody of the AEC directs the fusion glycoprotein or glycoprotein conjugate to specific sites in or on a tumor cell, and the enzyme portion of
25 the FUP or the AEC is capable of cleaving a prodrug to a tumor cytotoxic drug. As mentioned above, the modified carbohydrate complement enhances both the relative concentration of the FUP or AEC at the tumor site and increase the clearance of these proteins from non-
30 specific sites and from the general circulation.

Once the FUP or AEC has been substantially cleared from the plasma and the normal tissues, while remaining bound on the tumor, a prodrug (advantageously hydrophilic), which is non-toxic and which disseminates
35 extracellularly, is administered i.v. at appropriate (e.g., high) concentration. The prodrug is then cleaved

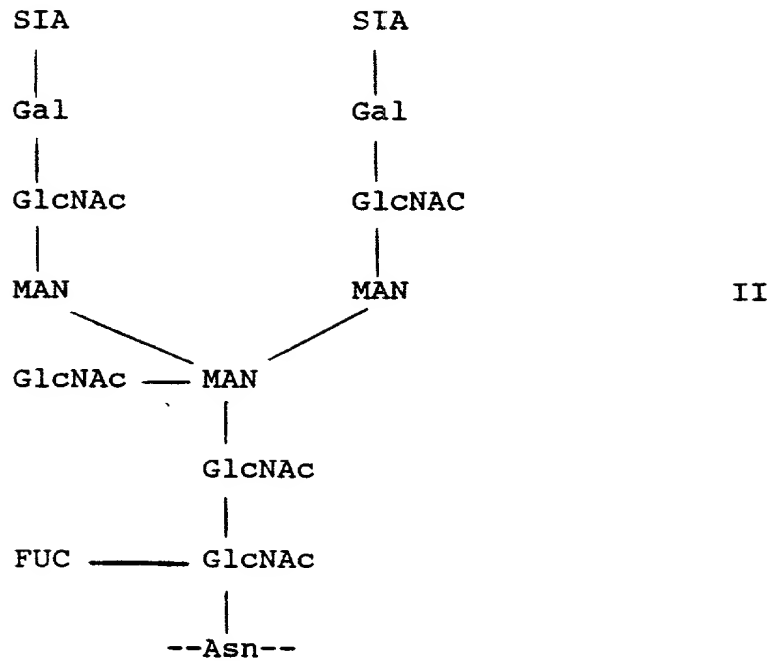
by the FUP or AEC which is bound to the tumor to yield a tumor cytotoxic drug, which is advantageously lipophilic.

Glycoproteins are composed of oligosaccharide units linked to the protein chain(s) either through the side chain oxygen atom of serine or threonine by O-glycosidic linkages, or to the side chain nitrogen of asparagine residues by N-glycosidic linkages. The sum total of oligosaccharide units of a glycoprotein is referred to as the carbohydrate complement. The N-linked oligosaccharides contain a common pentasaccharide core consisting of 3 mannose (MAN) and 2 N-acetylglucoseamine (GlcNAc) residues, as shown in Sketch I (high mannose type) below.



I

A complex type of oligosaccharide core is shown in Sketch II (see below), showing N-acetylneuraminic acid (sialic acid, SIA) residues as terminal carbohydrates, fucose (FUC) residues as side chains, and galactose (Gal) residues as penultimate sugars. Skilled artisans will appreciate that configurations other than those shown in sketches I and II are possible.



Additional sugars are attached to this common core in many different ways to form a great variety of oligosaccharide patterns. The nature of the terminal sugars in glycoproteins is part of a complex recognition system that is known to influence, *inter alia*, the uptake of glycoproteins by organs, macrophages and other tissues. See, e.g., Steer et al., *Prog. Liver Dis.*, 8:99 (1986); Stahl, *Curr. Opin. Immunol.*, 4: 49 (1992); Brady et al., *J. Inherit. Metab. Dis.*, in press (1994). These influences are highly tissue and glycoprotein specific, and it is not yet known *a priori* to predict patterns of enhanced clearance of particular circulating glycoproteins by specific tissues.

By galactosylating the FUP, or by eliminating terminal neuraminic acid residues from the protein by treating it with neuraminidase, the half life of the FUP in the plasma is shortened. It has been found, surprisingly, that the fusion protein which has been modified in this way continues to bind to the EAS, even at early time points, while retaining its specificity, avidity and enzymic activity. Further, the fusion protein is cleared from the plasma within 1-3 hours to

such an extent that efficient, tumor-selective activation of a suitable prodrug is effectively made possible without the need to inject a clearing second antibody as in WO 89/10140 and Sharma *et al.*, *Brit. J. Cancer* 61: 659 (1990). In addition, the present inventors have succeeded, by admixing, for example, galactose with the galactosylated FUP, in achieving still more efficient tumor localization. Further, it was possible successfully to extend these observations, within the scope of the invention, to additional FUPs and AECs, which were galactosylated or treated with neuraminidase, while preserving their biological properties.

Those skilled in the art will appreciate that different amounts of exposed residues may be utilized in the compounds of this invention. The number of exposed residues may be expressed as an average of exposed residues per molecule. For example, the average number of exposed residues per molecule generally will be at least about one, although averages of less than one are possible. Hence, for example, averages of less than 1, 1 to 2, 2 to 3, 3 to 4, 4 to 5, 5 to 6, 6 to 7, 7 to 8, 8 to 9, 9 to 10, 10 to 20, 20 to 50, 50 to 100, or greater than 100 are contemplated.

The general utility of the invention was verified using four different chemical compositions, namely, a xenogeneic antibody-enzyme conjugate, a humanized two-chain fusion protein, a humanized single-chain fusion protein and a xenogeneic single-chain fusion protein. Also useful are antibody fragment-enzyme conjugates, as well as to sFv-enzyme conjugates I and ligand-enzyme conjugates. The disclosure of WO 89/10140 is incorporated herein by reference to the extent that it discloses bifunctional proteins whose carbohydrate complement may be modified in accordance with this invention.

A representative AEC is composed of an intact monoclonal mouse antibody (e.g. as described in EP-A-0 388 914 which is incorporated by reference herein in its entirety) which is linked chemically to the enzyme E.

coli glucuronidase by means of a heterobifunctional reagent according to Haisma et al. (*Brit. J. Cancer* 66: 474 (1992)) or to Wang et al. (*Cancer Res.* 52: 4484 (1992)), which are incorporated herein by reference in their entirety. Additional linkage possibilities, which can likewise lead to functional AECs, have been summarized by Means et al (*Bioconjugate Chem.* 1: 2 (1990), which is also incorporated by reference.

A humanized, two-chain fusion protein is described in detail in EP-A-0 501 215. It is a protein which is composed of two polypeptide chains and which has been prepared by genetic manipulation. One chain was prepared by linking the nucleotide sequences that encode a humanized $V_H C_{H1}$ hinge S region to the nucleotide sequence which encodes a human β -glucuronidase (S = oligonucleotide encoding a polypeptide spacer). Following transfection and expression in suitable expression systems, preferably BHK or CHO cells, the nucleotide sequence which encodes the humanized $V_L C_L$ chain, together with the above-mentioned nucleotide sequence, produces the humanized two-chain fusion protein.

The humanized single-chain fusion protein was produced, following expression in suitable expression systems, preferably in BHK or CHO cells, by linking the nucleotide sequences which encode the humanized $V_H S V_L$ hinge S region (single chain Fv, sFv) and the nucleotide sequence which encodes human β -glucuronidase. The construction of a representative humanized single-chain fusion protein is described in Examples 1-4 below. A xenogeneic single-chain fusion protein is described in Example 5 below.

After recloning into suitable vectors, the constructs which are described in the examples below can also be expressed in other expression systems, such as, for example, *E. coli*, *Saccharomyces cerevisiae* and *Hansenula polymorpha*, insect cells or transgenic animals.

Non-human transgenic mammalian animals can be genetically engineered to secrete into readily accessible body fluids such as milk, blood and urine recombinant human FUPs of the invention in amounts and in forms that are suitable for treating humans with tumors.

The term "animal" here denotes all mammalian animals except humans. It also includes an individual animal in all stages of development, including embryonic and fetal stages. A "transgenic" animal is any animal containing cells that bear genetic information received, directly or indirectly, by deliberate genetic manipulation at the subcellular level, such as by microinjection or infection with recombinant virus.

"Transgenic" in the present context does not encompass classical crossbreeding or *in vitro* fertilization, but rather denotes animals in which one or more cells receive a recombinant DNA molecule. Although it is highly preferred that this molecule be integrated within the animal's chromosomes, the invention also contemplates the use of extrachromosomally replicating DNA sequences, such as might be engineered into yeast artificial chromosomes.

The term "germ cell line transgenic animal" refers to a transgenic animal in which the genetic information has been taken up and incorporated into a germ line cell, therefore conferring the ability to transfer the information to offspring. If such offspring, in fact, possess some or all of that information, then they, too, are transgenic animals.

The information to be introduced into the animal is preferably foreign to the species of animal to which the recipient belongs (i.e., "heterologous"), but the information may also be foreign only to the particular individual recipient, or genetic information already possessed by the recipient. In the last case, the introduced gene may be differently expressed than is the native gene.

The transgenic animals of this invention may be any, other than human, that produce milk, blood serum, and urine. Farm animals (pigs, goats, sheep, cows, horses, rabbits and the like), rodents (such as mice), and domestic pets (for example, cats and dogs) are included in the scope of this invention. It is preferred to select a transgenic animal that secretes into its milk a recombinant fusion protein, whose carbohydrate complement is modified to expose at least one mannose, galactose, N-acetylglucosamine, N-acetyllactose, glucose or fucose residue.

It is highly preferred that the transgenic animals of the present invention be produced by introducing into single cell embryos appropriate polynucleotides that encode the inventive FUPs in a manner such that these polynucleotides are stably integrated into the DNA of germ line cells of the mature animal and inherited in normal mendelian fashion.

Advances in technologies for embryo micromanipulation now permit introduction of heterologous DNA into fertilized mammalian ova. For instance, totipotent or pluripotent stem cells can be transformed by microinjection, calcium phosphate mediated precipitation, liposome fusion, retroviral infection or other means, the transformed cells are then introduced into the embryo, and the embryo then develops into a transgenic animal.

In a preferred method, developing embryos are infected with a retrovirus containing the desired DNA, and transgenic animals produced from the infected embryo. In a most preferred method, however, the appropriate DNAs are coinjected into the pronucleus or cytoplasm of embryos, preferably at the single cell stage, and the embryos allowed to develop into mature transgenic animals. Those techniques as well known. For instance, reviews of standard laboratory procedures for microinjection of heterologous DNAs into mammalian fertilized ova include: Hogan et al., *Manipulating the Mouse Embryo*, Cold Spring Harbor Press, 1986; Krimpenfort

et al., *Bio/Technology* 9: (1991); Palmiter et al., *Cell*, 41:343 (1985); Kraemer et al., *Genetic Manipulation of the Early Mammalian Embryo*, Cold Spring Harbor Laboratory Press, 1985; Hammer et al., *Nature*, 315:680 (1985); Meade et al., U.S. 4,873,316; Wagner et al., U.S. 5,175,385; Krimpenfort et al., U.S. 5,175,384, all of which are incorporated by reference in their entirety. The procedure of Meade et al., U.S. 4,873,316 is believed to provide one advantageous method of production, for example, using transgenic goats expressing the fusion protein under the control of the β -casein promoter in the mammary gland.

Genes for insertion into the genomes of transgenic animals so as to produce the FUPs of the invention can be obtained as described in the above-incorporated references and in the examples below. The gene encoding humanized two chain fusion glycoproteins are described in EP-A-0 501 215. The disclosure of which is incorporated herein by reference. The construction of a gene for a representative single-chain fusion glycoprotein is described in Examples 1-4 below. The gene for a single chain fusion glycoprotein is described in Example 5. Within the scope of the recombinantly produced modifications described herein, one can prepare constructs that include genes that encode proteins controlling posttranslational modification of expressed fusion glycoproteins. For example, constructs can be prepared, in which the sialyl transferase synthesis cycle is lacking or defective, thus producing fusion proteins in which terminal sialic acid residues are reduced in number or absent.

The cDNAs encoding desired FUPs can be fused, in proper reading frame, with appropriate regulatory signals to produce a genetic construct that is then amplified, for example, by preparation in a bacterial vector, according to conventional methods (see, Sambrook et al, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, 1989 which is incorporated herein by

reference in its entirety). The amplified construct is thereafter excised from the vector and purified for use in producing transgenic animals. Purification can be accomplished by means of one or more cycles of anionic HPLC; alternate techniques include ultracentrifugation through a sucrose or NaCl gradient, gel electrolution followed by agarose treatment and ethanol precipitation, or low pressure chromatography. Purification by several cycles of HPLC allows for remarkably high transformation frequencies, on the order of 20% or more in both mice and pigs.

The regulatory signals referred to above include *cis*-acting signals necessary for mammary gland-specific expression of the fusion proteins and their post-translational glycosylation, secretion of the expressed fusion glycoprotein into milk or other body fluids, and expression of full biological activity.

Such regulatory signals include the promoter that drives expression of the fusion genes. Highly preferred are promoters that are specifically active in mammary gland cells and that involve milk proteins. Among such promoters, preferred are those for the whey acidic protein (WAP), short and long α , β and kappa caseins, α -lactalbumin and β -lactoglobulin (BLG) promoters.

Promoters may be selected on the basis of the native protein compositions of the various animals' milks. For example, the WAP and BLG promoters are particularly useful with transgenic rodents, pigs and sheep.

The genes for these promoters have been isolated and characterized. Clark et al., *TIBTECH* 5:20 (1987); Henninghausen, *Protein Expression and Purification* 1:3 (1990), which are incorporated by reference. The promoters can be isolated by conventional restriction endonuclease and subcloning steps. A mouse WAP promoter, isolated as a 2.6 kb EcoR1-Kpn1 fragment immediately 5' to the WAP signal sequence can be used, although the "long" WAP promoter (the 5' 4.2 kb Sau 3A-Kpn1 promoter of the mouse gene is also suitable.

Important to the transgenic animal embodiment are regulatory sequences that direct secretion of proteins into milk and/or other body fluids. Generally, homologous or heterologous regulators sequences known to direct the secretion of milk proteins, such as either signal peptides from milk or nascent target polypeptides, can be used, although the scope of this invention includes signal sequences that direct the secretion of proteins into fluids other than milk.

Among the useful sequences that regulate transcription, in addition to those described above, are enhancers, splice signals, transcription termination codons, and polyadenylation sites.

The injected DNA sequences may also include a 3' untranslated region downstream of the DNA encoding the desired fusion protein, or the milk protein gene used for regulation. This region may stabilize the RNA transcript of the expression system and thus increase the yield of the desired fusion protein. Among these 3'untranslated regions useful in this regard are sequences that provide a poly A signal. Such sequences can be derived from, for example, the SV40 small t antigen, the casein 3' untranslated region, and others well known in this art.

Obtaining milk from transgenic female animals is done conventionally. McBurney et al., *J. Lab. Clin. Med.*, 64:485 (1964) ; Velandar et al., *Proc Natl. Acad. Sci. USA* 89: 12003 (1992).

Within the scope of recombinantly produced modifications, there are employed those, for example, in which the gene for sialyl transferase is inactive or is lacking, or in which other enzymes of the sialyl transferase synthesis cycle are deficient or are lacking. Other preferred expression systems exhibit overexpression of galactosyl transferase or mannose-6-phosphate synthetases/transferases. In addition, it has been found that clones which have been produced from CHO cells having a very high ability to express fusion protein, for example, by means of double selection, in accordance with

EP-A-0 330 977 (which is incorporated by reference herein in its entirety), are deficient in sialylation. Such clones, that may generally be produced by a process known as "homologous recombination" (Pomerantz et al., Progress in Cancer Res. and Therapy, 30:37-45 (1975), incorporated by reference herein in its entirety), are thus very suitable for use as expression systems.

These proteins (antibody-enzyme conjugate, humanized two-chain fusion protein, humanized single-chain fusion protein and xenogeneic single-chain fusion protein, which have been described by way of example), were, once they had been purified by anti-idiotypic and/or anti- β -glucuronidase immunaffinity chromatography, chemically galactosylated in accordance with the method described by Krantz et al. (Biochemistry 15: 3963 (1976) which is incorporated by reference herein in its entirety) or, alternatively, treated with carrier-bound neuraminidase. In that which follows, they are termed modified glycoproteins.

The modified proteins were compared *in vitro* and *in vivo* to the control unmodified starting proteins which had been expressed in BHK cells. The *in vitro* tests for specificity, affinity, quantitative immunoreactivity and quantitative enzyme activity demonstrated that the modified proteins did not differ significantly in these respects from the control proteins. In contrast, the half life ($t_{1/2\beta}$) of the modified proteins in mouse and rat plasma (*in vivo*) was dramatically shortened (Tables 6, 7).

As a result of this dramatic shortening of the $t_{1/2\beta}$, at 1-3 hours after injecting the galactosylated proteins i.v. into tumor-bearing nude mice, modified proteins could no longer be detected in the plasma. In the case of the desialylated proteins, the $t_{1/2\beta}$ was shortened to such an extent that desialylated protein was no longer detectable in the plasma after 48 hours. At the same time, the concentration of functionally active modified proteins in the tumor was in the range from 200-400 ng/g

of tumor (a very high specificity ratio $> 100:1$ was consequently obtained on injecting $\approx 400 \mu\text{g}$ of modified protein per mouse).

5 Viewed in absolute terms, the concentrations of modified proteins can be two to three times higher than those which are achieved, after appreciably longer times, for a comparable specificity ratio using unmodified starting proteins *in vivo*. Furthermore, the above-mentioned high specificity ratio (μg of modified protein/g of tumor: μg of modified protein/g of normal tissue) for modified proteins, is attained after only a few hours (1-3 hours or 48 hours, respectively), whereas, in the case of the unmodified starting proteins, a comparable specificity ratio (μg of unmodified starting protein/g of tumor: μg of unmodified starting protein/g of normal tissue) is only reached after several days (7-8 days), or even requires the use of a second antibody to accelerate the rate of clearance from normal tissue.

20 The rapid removal of the modified proteins from the plasma and the extracellular region of the organism by means of internalization via sugar-binding receptors (chiefly the galactose receptor in the liver, Thornburg et al., *J. Biol. Chem.* 255: 6820 (1980)) should also lead to the modified proteins having reduced immunogenicity in humans, particularly in the case of the antibody-enzyme conjugate and the xenogeneic single-chain fusion protein. This therefore also facilitates the use of xenogeneic or humanized FUPs in anti-tumor therapy, or at the least makes such use appear feasible for the first time.

30 A particularly useful humanized two-chain fusion protein has been expressed in CHO cells that had been selected for a very high level of expression, and purified by anti-idiotypic affinity chromatography. Three or seven days after i.v. injection, this FUP was concentrated in the tumor to an extent 2-3-fold higher than that of the analogous fusion protein that is expressed in the BHK cells (Table 8). In addition, the

FUP that has been expressed in CHO cells is removed from the plasma appreciably more efficiently than the fusion protein expressed in BHK cells, so that tumor: plasma ratios of > 15 are reached by day 3 in the case of the CHO fusion protein. In the case of the BHK fusion protein, the corresponding ratios are < 1 (Table 8). On day 7, the tumor:plasma ratios for the CHO fusion protein are in the region of 130 while those for the BHK fusion protein are in the region of 20 (Table 8).

These highly significant pharmacokinetic differences between the humanized two-chain fusion protein expressed in CHO cells or expressed in BHK cells can be explained by differences in the carbohydrate content of the fusion proteins. An analysis of the monosaccharide components in the carbohydrate content of the fusion protein expressed in BHK or CHO cells is given in Table 1a. Differences are observed mainly in the content of galactose, mannose and N-acetylneuraminic acid.

Table 1a

An analysis of the monosaccharide components in the carbohydrate content of the fusion proteins

mol monosaccharide / mol fusion protein monomer (125kDa)					
	Fucose	N-acetyl glucosamine	Galactose	Mannose	N-acetyl neuraminic acid
CHO fusion protein	0.6	4.35	1.40	7.04	0.54
BHK fusion protein	0.68	4.46	1.59	6.31	0.69

Method:

Neuraminic acid was determined by the method of Hermentin and Seidat (1991) GBF Monographs Volume 15, pp. 185-188 (after hydrolysis of 30 min in the presence of 0.1 N sulfuric acid at 80° C and an subsequent

neutralization with 0.4 N sodium hydroxide solution) by high-pH anion exchange chromatography with pulsed amperometric detection (HPAE-PAD).

5 The monosaccharide components were determined (after hydrolysis of 4 h in the presence of 2 N trifluoroacetic acid at 100° C and evaporation to dryness in a SpeedVac) likewise by HPAE-PAD in a motivation of the method described by Hardy et al. (1988) *Analytical Biochemistry* 170, pp. 54-62.

10 Particularly, the increased amounts of mannose or mannose-6-phosphate in combination with reduced amounts of N-acetylneuraminic acid, as observed in the fusion protein expressed in CHO cells, might be responsible for its faster elimination from plasma and normal tissues due
15 to more efficient binding to mannose and galactose receptors (compare Table 8, pharmacokinetics of unmodified fusion proteins). Furthermore, glycan mapping showed higher contents of high mannose/asialo-structures (see Sketch II above) in the CHO-expression product
20 compared to the normal BHK-expression product.

The high β -glucuronidase concentrations, which were determined in the enzyme activity test, represent the activity of the endogenous murine β -glucuronidase and that of the FUP, as well as that of any human β --
25 glucuronidase which may have been liberated from the latter by the cleavage which can potentially occur. Using enzyme-histochemical methods (Murray et al., *J. Histochem. Cytochem.* 37: 643 (1989)), it was demonstrated that this enzyme activity was present as
30 intracellular activity in the normal tissues. Thus, this catalytic potential either does not contribute, or only contributes unimportantly, to the cleavage of a hydrophilic prodrug which is disseminated extracellularly.

35 The several embodiments exemplified below are not to be taken as in any way limiting the scope of the invention which is described in the specification and in the appended claims.

EXAMPLES

Examples 1 - 4:

Recombinant preparation of a humanized single-chain fusion protein from a humanized tumor antibody moiety and human β -glucuronidase.

Example 1

Using the oligonucleotides pAB-Back and Linker-Anti (Table 1), the V_H gene, including its own signal sequence, is amplified from pABstop 431/26 hum V_H (Güssow et al, 1991, above). Using the oligonucleotides Linker-Sense and V_L (Mut)-For (Table 2), the V_L gene is amplified (Fig. 1) from pABstop 431/26 hum V_L (Güssow et al., 1991, above).

Table 1

pAB-Back: SEQUENCE ID NO. 1

5' 3'
ACC AGA AGC TTA TGA ATA TGC AAA TC

Linker-Anti: SEQUENCE ID NO. 2

5'
GCC ACC CGA CCC ACC ACC GCC CGA TCC ACC GCC TCC TGA
3'
GGA GAC GGT GAC CGT GGT C

Table 2

Linker-Sense: SEQUENCE ID NO. 3

5'
GGT GGA TCG GGC GGT GGT GGG TCG GGT GGC GGC GGA TCT
3'
GAC ATC CAG CTG ACC CAG AGC

V_L (Mut)-For: SEQUENCE ID NO. 4

5'
TGC AGG ATC CAA CTG AGG AAG CAA AGT TTA AAT TCT ACT
3'
CAC CTT TGA TC

Example 2

The oligonucleotides Linker-Anti and Linker-Sense are partially complementary to each other and encode a polypeptide linker which is intended to link the V_H and V_L domains to form an sFv fragment. In order to fuse the amplified V_H and V_L fragments, they are purified and introduced into a 10-cycle reaction as follows:

H_2O :	37.5 μ l
dNTP's (2.5 mM)	5.0 μ l
PCR buffer (10x)	5.0 μ l
Taq polymerase (Perkin-Elmer Corp., Emmeryville, CA) (2.5 U/ μ l)	0.5 μ l
0.5 μ g/pl DNA of the V_L frag.	1.0 μ l
0.5 μ g/pi DNA of the V_H frag.	1.0 μ l

PCR buffer (10x): 100 mM Tris, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.1% (w/v) gelatin.

The surface of the reaction mixture is sealed off with paraffin and the 10-cycle reaction is subsequently carried out in a PCR apparatus using the program 94°C, 1 min; 55°C, 1 min; 72°C, 2 min. After that, 2.5 pM of the flanking primers pAB-Back and V_L (Mut)-For are added and a further 20 cycles are carried out. A PCR fragment is obtained which is composed of the V_H gene, which is connected to the V_L gene via a linker (Fig. 4). The V_H gene's own signal sequence is also located prior to the V_H gene. As a result of using the oligonucleotide V_L (Mut)-For, the last nucleotide base of the V_L gene, a C, is at the same time replaced by a G. This PCR fragment encodes a humanized single-chain Fv (sFv).

Example 3

The sFv fragment from Example 2 is restricted with HindIII and BamHI and ligated into the vector pABstop 431/26 V_H hu β gluc1H, which has been completely cleaved with HindIII and partially cleaved with BgIII. The vector pABstop 1/26 V_H hu β gluc1H contains a V_H exon, including the

V_H-specific signal sequence, followed by a CH1 exon, the hinge exon of a human IgG3 C gene and the complete cDNA of human β -glucuronidase. The plasmid clone pMCG-E1 is isolated, which clone contains the humanized sFv 431/26, a hinge exon and the complete β -glucuronidase (Fig. 3a). Vector pABstop 431/26V_Hhu β gluc is described in Bosslet et al., *Brit. J. Cancer* 65: 234 (1992), which is incorporated by reference herein in its entirety and where information on the remaining individual components can be obtained from the references listed therein.

Example 4

The clone pMCG-E1 is transfected, together with the plasmid pRMH 140 (Fig. 4), which carries a neomycin resistance gene, and the plasmid pSV2 (Fig. 5), which carries a methotrexate resistance gene, into BHK cells. The BHK cells then express a fusion protein which possesses both the antigen-binding properties of Mab BW 431/26hum and the enzymic activity of human β -glucuronidase (see Examples 8 and 9).

Example 5

Construction of xenogeneic single-chain fusion protein

The xenogeneic single-chain fusion protein was produced, following expression in suitable expression systems, preferably in BHK cells, by linking the nucleotide sequences which encode the humanized V_H, S, V_L hinge and S regions (see Examples 1-4 and below) to the nucleotide sequence which encodes *E. coli* β -glucuronidase. The construction of a single-chain fusion protein from a humanized sFv (antiCEA) and *E. coli* β -glucuronidase is described in detail below.

The sFv 431/26 fragment (a) is employed as the template for a PCR using the oligos pAB-Back (Table 1) and sFv-For (Table 3). In this way, BgIII and HindIII cleavage sites are introduced at the 3' end of the newly generated sFv 431/26 fragment (b). The PCR fragment is purified and digested with HindIII, and then ligated into

a pUC18 vector which has been cut with *Hind*III and treated with alkaline phosphatase. The plasmid clone pKBO1 is isolated, containing the sFv fragment with the BglII cleavage site (Fig. 6).

5 The gene encoding the *E. coli* (β -glucuronidase is amplified from the vector pRAJ260 (Jefferson et al., *Proc. Natl. Acad. Sci. USA*, 83:8447 (1986)) by PCR using the oligos *E. coli* (β -gluc-Back1 (Table 4) and *E. coli* β -gluc-For (Table 5), and at the same time provided at the
10 5' end with a *Bg*III cleavage site, at the 3' end with an *Xba*I cleavage site and, additionally at the 5' end, with a sequence encoding a linker. The resulting fragment is purified and digested with *Bg*III/*Xba*I, and then cloned into the vector pKBO1 which has likewise been digested
15 with *Bg*III/*Xba*I. The plasmid clone pKBO2 is isolated, containing sFv 431/26 linked to *E. coli* β -glucuronidase via a linker sequence (Fig. 7).

The sFv-*E. coli* β -gluc. fragment, obtained from vector pKBO2 by *Hind*III/*Xba*I digestion, is purified and
20 then ligated into the expression vector pABstop (Zettlmeissl et al., *Behring Institute Mitteilungen (Communications)* 82: 26 (1988)) which has likewise been cut with *Hind*III/*Xba*I. The plasmid clone pKBO3 is isolated, containing the humanized sFv 431/26, a linker
25 and the complete *E. coli* β -glucuronidase (Fig. 8).

Table 3

sFv For: SEQUENCE ID NO. 5

5' 3'
TTT TTA AGC TTA GAT CTC CAC CTT GGT C

Table 4

E. coli β -gluc-Back 1: SEQUENCE ID NO. 6

5'
AAA AAG ATC TCC GCG TCT GGC GGG CCA CAG TTA CGT GTA GAA
3'
35 ACC CCA

E. coli β -gluc-For: SEQUENCE ID NO. 7

[illegible]

Tab.6

**Pharmacokinetic comparison between unmodified and modified humanized two-chain fusion proteins
in CD-1 nude mice bearing a human tumor xenograft (MzSto1)**

unmodified fusion protein produced in BHK cells												unmodified fusion protein produced in BHK cells											
μg fusion protein /g of tumor or /g of organ measured in an OFAT												μg of β -glucuronidase /g of tumor or /g of organ measured in an EAT											
	tumor	spleen	liver	intestine	kidney	lung	heart	plasma		tumor	spleen	liver	gut	kidney	lung	heart	plasma						
0.05 hr	3	21	61.4	6.5	35.6	77.6	60.1	456.2		n.d.	48.5	81.7	18.6	45	83.7	60.2	n.d.						
1 hr	4.9	15	26.1	8.6	17.3	33.9	19.5	199.9		n.d.	58.9	126.2	20	24.1	39.7	21.1	n.d.						
1.5 hr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.						
2 hr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.						
3 hr	5.7	4.8	14.8	3.5	6.5	7.7	2.5	122		n.d.	50.2	125.2	15.6	11.4	13.3	4.2	n.d.						
5.5 hr	3.8	3.8	8.4	3.8	7.7	8.8	2.9	84.9		n.d.	78	177.8	17	9.3	14.5	5.4	n.d.						
24 hr	4.7	1	2.1	0.6	2.5	2.1	0.5	19		n.d.	90.7	267.5	15.9	6.7	8.5	4	n.d.						
48 hr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.						
168 hr	0.19	0.005	0.003	0	0	0.002	0	0		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.						

modified fusion protein produced in BHK cells (galactosylated)												modified fusion protein produced in BHK cells (galactosylated)											
μg galactosylated fusion protein /g of tumor or /g of organ measured in an OFAT												μg of β -glucuronidase /g of tumor or /g of organ measured in an EAT											
	tumor	spleen	liver	intestine	kidney	lung	heart	plasma		tumor	spleen	liver	gut	kidney	lung	heart	plasma						
0.05 hr	1.6	7.1	51.9	2.15	7.78	21.6	15.7	83.3		n.d.	35.5	75.1	14	13	39	17.9	n.d.						
1 hr	0.5	0.19	1.9	0.29	1.16	0.26	0.17	0.06		n.d.	27.6	167.5	17.4	5.5	7.2	1.4	n.d.						
1.5 hr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.						
2 hr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.						
3 hr	0.16	0.27	0.09	0.03	0	0.3	0	0		n.d.	23.8	132.7	8.9	4.4	6.3	0.86	n.d.						
5.5 hr	0.27	0.02	0.11	0.05	0.02	0.08	0	0		n.d.	28.3	164.5	10.7	4.1	6	1.1	n.d.						
24 hr	0.05	0.02	0.04	0	0	0	0	0		n.d.	31.5	126.2	12.8	4.7	5.6	1.4	n.d.						
48 hr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.						
168 hr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.						

Tab. 6-continuation

Tab.6

modified fusion protein produced in BHK cells (desialylated)										modified fusion protein produced in BHK cells (desialylated)									
μg desialylated fusion protein /g of tumor or /g of organ										μg of β - glucuronidase /g of tumor or /g of organ									
measured in an OFAT										measured in an EAT									
	tumor	spleen	liver	Intestine	kidney	lung	heart	plasma		tumor	spleen	liver	gut	kidney	lung	heart	plasma		
0.05 hr	1.3	14.6	38.9	10.2	20.6	49.8	24.2	234.9		1.3	43.2	63.2	48.7	23.8	56.1	25.7	250		
1 hr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
1.5 hr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
2 hr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
3 hr	3.3	1.1	2.8	0.37	1.5	1.8	1.3	13.8		3.3	69.8	199.2	9.2	7.4	6.1	3.9	13.5		
5.5 hr	2.9	0.1	0.22	0.02	0.04	0.19	0.06	0.65		2.9	68.7	342.1	10.6	4	4.1	2.5	0.76		
24 hr	0.4	0.1	0.04	0.002	0.004	0.16	0.001	0.004		0.4	179	641.1	34.6	15.8	16.9	7	0.058		
48 hr	0.1	0	0.04	0	0	0.55	0	0		0.1	185.9	639.7	32.7	8.8	21	12.7	0.013		
168 hr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		

- 28 -

[illegible]

Tab. 8

Pharmacokinetic comparison between unmodified humanized two-chain fusion proteins, produced in BHK cells and CHO cells, in CD-1 nude mice bearing a human tumor xenograft (MzSto1)

unmodified fusion protein produced in BHK cells										unmodified fusion protein produced in BHK cells							
μg of fusion protein /g of tumor or /g of organ measured in an OFAT										μg of β -glucuronidase /g of tumor or /g of organ measured in an EAT							
	tumor	spleen	liver	intestine	kidney	lung	heart	plasma		tumor	spleen	liver	intestine	kidney	lung	heart	plasma
0.05 hr	3.807	22.779	44.411	7.732	27.792	53.59	33.941	413		6.473	27.927	44.748	13.045	27.419	59.345	30.897	n.d.
3hr	6.166	8.847	20.099	4.125	12.609	26.363	12.93	147		9.982	70.009	445.32	15.714	16.415	27.014	14.427	n.d.
24hr	4.944	0.935	1.416	0.249	1.315	2.779	1.493	12.3		28.384	41.799	33.834	6.605	2.422	3.99	2.498	n.d.
72hr	0.818	0.094	0.14	0.058	0.112	0.241	0.136	1.152		9.929	20.458	12.391	12.416	1.051	2.283	1.306	n.d.
168hr	0.314	0.002	0.005	0.002	0.002	0.008	0	0.015		7.423	7.393	6.083	5.164	0.94	1.483	0.502	n.d.

unmodified fusion protein produced in CHO cells										unmodified fusion protein produced in CHO cells									
μg of fusion protein /g of tumor or /g of organ measured in an OFAT										μg of β -glucuronidase /g of tumor or /g of organ measured in an EAT									
tumor	spleen	liver	intestine	kidney	lung	heart	plasma	tumor	spleen	liver	intestine	kidney	lung	heart	plasma				
0.05 hr	3.583	19.179	33.392	7.96	23.089	61.279	24.018	308	6.475	23.883	29.696	14	23.223	56.864	23.042	n.d.			
3hr	6.526	8.555	17.787	7.098	11.613	26.755	10.824	157.9	10.96	89.594	204.82	15.928	13.66	26.994	12.556	n.d.			
24hr	4.668	1.002	1.225	0.299	1.218	2.926	1.361	12.082	29.279	72.999	23.754	7.011	2.513	5.003	3.089	n.d.			
72hr	2.176	0.036	0.028	0.013	0.023	0.059	0.029	0.144	50.13	25.828	7.527	7.613	1.542	2.568	1.719	n.d.			
168hr	0.653	0.003	0.002	0.003	0	0.003	0	0.005	5.515	14.251	4.172	4.172	1.202	1.588	1.193	n.d.			

Example 6

Galactosylation of the two-chain fusion protein

5 The galactosylation of the fusion protein was carried out using a modification of the method of Mattes (*J. Natl. Cancer Inst.*, 79: 855 (1987) which is incorporated herein in its entirety):

10 Cyanomethyl-2,3,4,6-tetra-O-acetyl-1-thio- β -D-galactopyranoside (Sigma; 250 mg) was dissolved in dried methanol (Merck; 6.25 ml), and 625 μ l of a methanolic sodium methoxide solution (5.4 mg/ml) were then pipetted in. After incubating at room temperature for 48 h, an aliquot of 5 ml of the activated galactose derivative was removed and the methanol evaporated off in a stream of nitrogen 100 ml of a fusion protein solution (1 mg/ml in 15 0.25 M sodium borate buffer, pH 8.5) were added to the remaining residue, and the mixture incubated at R.T. for 24 h. This was followed by dialysis overnight against PBS.

20 Galactosylation of the preformed BW 431/26-E. coli β -glucuronidase conjugates and the monoclonal antibody BW 431/26 was carried out in a similar manner. Using similar chemistry, lactosilation, N-acetyl-lactosilation and glucosilation of AEC and FUP can be performed.

Example 7

Working up organs/tumors for FUP determination

25 The following sequential steps were carried out:

1. Nude mice (CD1), which possess a subcutaneous tumor and which have been treated with fusion protein or antibody-enzyme conjugate, are bled retroorbitally and 30 then sacrificed.

2. The blood is immediately added to an Eppendorf tube which already contains 10 μ l of Liquemin 25000 (from Hoffman LaRoche AG).

35 3. The treated blood from 2. above is centrifuged (in a Megafuge 1.0 centrifuge, from Heraeus) at 2500 rpm for 10 min; the plasma is then isolated and frozen down until testing.

4. The organs, or the tumor, are removed, weighed and then completely homogenized with 2 ml of 1% BSA in PBS, pH 7.2.

5. The tumor homogenates are adjusted to pH 4.2 with 0.1N HCl (the sample must not be overtitrated, or the β -glucuronidase will be activated prematurely at pH < 3.8!)

6. Homogenates are centrifuged at 16000 g for 30 min; the clear supernatant fluids are removed and neutralized with 0.1 N NaOH.

7. The supernatants and the plasma can now be tested in an OFAT (measures FUP concentration) or an EAT (measures β -glucuronidase concentration), as described in the examples below.

Example 8

OFAT (organ fusion protein activity test)

The test proceeds in the following manner:

1. 75 μ l of a goat anti-human-kappa antibody (from Southern Biotechnology Associates, Order No. 2060-01), diluted 1:300 in PBS, pH 7.2, are added to each well of a microtitration plate (polystyrene U form, type B, from Nunc, Order No. 4-60445).

2. The microtitration plates are covered and incubated at room temperature overnight.

3. The microtitration plates are then washed 3 times with 250 μ l of 0.05 M Tris-citrate buffer, pH 7.4, per well.

4. These microtitration plates, which have been coated in this manner, are incubated with 250 μ l of blocking solution (1% casein in PBS, pH 7.2) per well at room temperature for 30 mins (blocking of non-specific binding sites)

(coated microtitration plates which are not required are dried at room temperature for 24 hours and then sealed, together with desiccator cartridges, in coated aluminium bags for long-term storage).

5. The substrate is prepared while the blocking is proceeding (fresh substrate for each test: 2.5 mM 4-

methyumbelliferyl β -D-glucuronide, Order No.: M-9130, from Sigma, in 200 mM Na acetate + 0.01% BSA, pH 4.5).

5 6. Thereafter, 10 samples + 1 positive control + 1 negative control are diluted in 1% casein in PBS, pH 7.2, 1:2 in 8 steps (starting from 150 μ l of sample, 75 μ l of sample are pipetted into 75 μ l of casein recipient solution, etc.) in an untreated 96-well U-shape bottomed microtiter plate (polystyrene, from Renner, Order No. 12058).

10 7. The blocking solution is sucked off from the microtitration plate coated with anti-human-kappa antibody, and 50 μ l of the diluted samples are transferred to each well of the test plate from the dilution plate, and the test plate is incubated at room temperature for 30 min.

15 8. The test plate is washed 3 times with ELISA washing buffer (Behringwerke, Order No. OSEW96);

9. 50 μ l of substrate are applied per well and the test plates are covered and incubated at 37°C for 2 h.

20 10. 150 μ l of stock solution (0.2 M glycine + 0.2% SDS, pH 11.7) are then added to each well.

25 11. Fluorometric evaluation is carried out in a Fluoroscan II (ICN Biomedicals, Cat. No. 78-611-00) at an excitation wavelength of 355 nm and an emission wavelength of 460 nm.

30 12. With the aid of the fluorescence values for the positive control (dilution series with purified fusion protein as the standard curve) included in the identical experiment, the unknown concentration of fusion protein is determined in the sample.

Example 9: EAT (enzyme activity test)

The test is carried out in the following manner:

35 1. 10 samples + 1 positive control + 1 negative control are diluted 1:2 in 1% casein in PBS, pH 7.2, in 8 dilution steps in a 96-well microtiter plate (polystyrene, from Renner, Order No. 12058) so that each well contains 50 μ l of sample.

2. 50 μ l of substrate (2.5 mM 4-methylumbelliferyl β -D-glucuronide (from Sigma, Order No. M-9130, in 200 mM Na acetate + 0.01% BSA, pH 4.5) are added to each well.

3. The microtiter plate is covered and incubated at 37°C for 2 h.

4. 150 μ l of stock solution (0.2 M glycine + 0.2% SDS, pH 11.7) are then added per well.

5. Fluorometric evaluation is carried out in a Fluoroscan II (ICN Biomedicals, Cat. No. 78-611-00) at an excitation wavelength of 355 nm and an emission wavelength of 460 nm;

6. With the aid of the positive control (dilution series with purified fusion protein as the standard curve) which has been included, the sample concentrations can now be calculated.

Example 10

Desialylation of the two-chain fusion glycoprotein

The two-chain fusion protein was desialylated according to Murray (*Methods in Enzymology* 149: 251 (1987)). Eight units of neuraminidase (Sigma, type X-A from *Clostridium perfringens*) coupled to agarose were washed 3x with 40 ml of 100 mM sodium acetate buffer, pH 5, and then taken up as a 1:1 suspension. One hundred milliliters of two-chain fusion protein (1 mg/ml in sodium acetate buffer, pH 5) were added to this suspension, which was then incubated with gentle shaking at 37°C for 4 h. The immobilized neuraminidase was removed by centrifuging off, and the fusion protein was dialyzed overnight against PBS.

Example 11

Demonstrating rapid elimination of modified FUP

100 mg of humanized two-chain fusion glycoprotein were purified from BHK transfectant supernatant, as described in EP-A-0 501 215, pages 10-11. The purified protein was galactosylated or desialylated, as described in the preceding examples.

400 μ g of the modified protein thus obtained, in this case the galactosylated humanized two-chain fusion protein, were injected i.v. into nude mice. The mice had been injected subcutaneously, 10 days previously, with 5 10^6 CEA-expressing human stomach carcinoma cells (Mz-Sto-1). At various time intervals, the mice were killed, and the concentration of functionally active modified protein was determined in the tumor, the plasma and the normal tissues using the OFAT or the EAT (see Examples 7, 8 and 10 9).

Nude mice which in each case had been provided with 1 x 10^6 CEA-negative human tumors (Oat-75) were used as the antigen control. In addition, identical quantities of the humanized two-chain fusion protein (starting 15 protein) or of a humanized two-chain fusion protein sample which had been treated with solid phase neuraminidase (desialylated protein) were injected i.v.. as the protein control (see Example 10). The quantities of the functionally active proteins found in the organs 20 in this representative experiment are given in Tables 6 and 7.

Comparable results were found in the identical animal model system using the example of a CEA-positive colon carcinoma, a CEA-positive rectal carcinoma, a CEA- 25 positive adenocarcinoma of the lung, a CEA-positive pancreatic carcinoma, a CEA-positive thyroid gland carcinoma, and a CEA-positive mammary carcinoma.

Therapeutic effects which are superior to those of the standard chemotherapy can be achieved when suitable 30 non-toxic prodrugs, e.g. those described in EP-A-0 511 917, are used which are applied at a point in time at which the modified proteins have been largely eliminated from the plasma or have been internalized and degraded in the normal tissues. These effects can be improved still 35 further by adding large quantities of galactose to the relevant modified protein, leading to optimization of the pharmacokinetics.

Further improvements can be achieved in accordance with the method described by Jähde et al. (*Cancer Res.* 52: 6209 (1992)) by adding glucose, phosphate ions or metaiodobenzylguanidine to the relevant modified protein, or injecting these compounds prior to the protein. This method leads to a decline in the pH within the tumor. This results in more efficient catalysis of the prodrugs by the enzymes used in the modified and non-modified proteins according to the invention. Alternatively, HCO_3^- can also be employed for lowering the pH in the tumor (Gullino et al., *J. Nat. Cancer Inst.* 34: 857, (1965)).

It will be apparent to those skilled in the art that various modifications and variations can be made to the compositions and processes of this invention. Thus, it is intended that the present invention cover such modifications and variations, provided they come within the scope of the appended claims and their equivalents.

The disclosure of all publications cited above are expressly incorporated herein by reference in their entireties to the same extent as if each were incorporated by reference individually. The disclosure of German Patent Application P 43 14 556.6 for which benefit under 35 USC §119 is claimed, is expressly incorporated herein in its entirety.

What is claimed is:

1. A compound comprising a bifunctional fusion glycoprotein or bifunctional glycoprotein conjugate, the compound comprising a carbohydrate complement, and:
 - a. at least one first portion which possesses enzymatic activity;
 - b. at least one second portion which binds specifically to an epitope of a tumor-specific antigen; wherein the carbohydrate complement comprises at least one exposed terminal carbohydrate residue selected from the group consisting of mannose, galactose, N-acetylglucosamine, N-acetyllactose, glucose and fucose.
2. A compound as claimed in claim 1, wherein the exposed carbohydrate residue is produced by enzymatic degradation.
3. A compound as claimed in claim 2, wherein the enzymatic degradation is effected by an enzyme selected from the group consisting of endoglycosidases, exoglycosidases, and neuraminidases, and a combination thereof.
4. A compound as claimed in claim 1, wherein the exposed carbohydrate residue is produced by chemical degradation.
5. A compound as claimed in claim 1 wherein the exposed carbohydrate residue is added to the compound by chemical means.
6. A compound as claimed in claim 1, wherein the first portion consists essentially of an enzyme.

7. A compound as claimed in claim 6, wherein the enzyme is selected from the group consisting of penicillin G amidase, penicillin V amidase, β -lactamase, alkaline phosphatase, carboxypeptidase G2, carboxypeptidase A, cytosine deaminase, nitroreductase, diaphorase, arylsulfatase, glycosidase, β -glucosidase, and β -glucuronidase.

8. A compound as claimed in claim 6 wherein the enzyme is a catalytic antibody.

9. A compound as claimed in claim 1, wherein the tumor cell marker to which the second portions binds comprises a tumor associated antigen selected from the group consisting of CEA, N-CAM, N-cadherin, PEM, GICA, TAG-72, TF β , GM3, GD3, GM2, GD2, GT3, HMWMAA, pMel17, gp113 (Muc18), p53, p97, MAGE-1, gp105, erbB2, EGF-R, PSA, transferrin-R, P-glycoprotein and cytokeratin.

10. A compound as claimed in claim 1, wherein the second portion consists essentially of an antibody or a fragment thereof.

11. A compound as claimed in claim 11, wherein the antibody is the monoclonal antibody BW 431/26 or a fragment thereof.

12. A compound as claimed in claim 1, wherein the first portion and the second portion are connected by a linker molecule.

13. A compound of claim 12 having the formula huTuMab-L- β -Gluc, wherein huTuMab is a human tumor specific monoclonal antibody or a tumor binding fragment thereof, L is the linker molecule and β -Gluc is a human β -glucuronidase.

14. A compound as claimed in claim 1, comprising a fusion glycoprotein that has been synthesized in CHO cells, the cells having been selected for a high level of expression of the glycoprotein.

15. A compound as claimed in claim 1, wherein the exposed carbohydrate is a galactose or a mannose.

16. A pharmaceutical preparation containing a compound as claimed in claim 1 in a pharmaceutically acceptable vehicle.

17. A pharmaceutical preparation containing a compound as claimed in claim 1, and an agent capable of lowering the pH in a tumor to be treated, in a pharmaceutically acceptable vehicle.

18. A pharmaceutical preparation, containing a compound as claimed in claim 1, and galactose, in a pharmaceutically acceptable vehicle.

19. A method of treating a tumor in a subject, comprising the steps of:

a. administering in a first step a pharmaceutical preparation as claimed in claim 16 to a subject having a tumor;

b. administering in a second step a non-toxic prodrug that will subsequently be cleaved into a cytotoxic drug at the site of the tumor by the first portion, so that the tumor will regress thereby.

a. preparing a DNA encoding a fusion glycoprotein according to claim 1;

b. inserting the DNA in an expression vector;

d. isolating the expressed fusion glycoprotein.

21. A process according to claim 20, wherein the expression system is a transgenic non-human mammalian animal.

Abstract of the Disclosure

Provided herein are carbohydrate complement-modified bifunctional glycoproteins, and their use in tumor-selective therapy. The bifunctional glycoproteins
5 comprise a first component that specifically binds to a tumor-specific antigen and a second component having enzymatic activity by means of which a non-toxic prodrug is cleaved into a cytotoxic drug. The carbohydrate complement comprises at least one exposed carbohydrate
10 residue selected from the group consisting of mannose, galactose, N-acetylglucosamine, N-acetyllactose, glucose and fucose. The modified carbohydrate complement contributes to increased relative concentration of the glycoproteins at the site of the tumor, and enhanced
15 clearance from the general circulation and non-tumor sites.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Bosslet, Klaus
Czech, Joerg
Hoffmann, Dieter
- (ii) TITLE OF INVENTION: BIFUNCTIONAL GLYCOPROTEINS HAVING A
MODIFIED CARBOHYDRATE COMPLEMENT, AND THEIR USE IN
TUMOR-SELECTIVE THERAPY
- (iii) NUMBER OF SEQUENCES: 7
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Foley & Lardner
 - (B) STREET: 3000 K Street, N.W., Suite 500
 - (C) CITY: Washington, D.C.
 - (E) COUNTRY: USA
 - (F) ZIP: 20007-5109
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/663,406
 - (B) FILING DATE: 13-JUN-1996
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/235,395
 - (B) FILING DATE: 29-APR-1994
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: EP 94106394.3
 - (B) FILING DATE: 25-APR-1994
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: DE P 43 14 556.1
 - (B) FILING DATE: 04-MAY-1993
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: SANDERCOCK, Colin G.
 - (B) REGISTRATION NUMBER: 31,298
 - (C) REFERENCE/DOCKET NUMBER: 58315/118/BEAK
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (202)672-5300
 - (B) TELEFAX: (202)672-5399
 - (C) TELEX: 904136

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ACCAGAAGCT TATGAATATG CAAATC

26

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 58 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCCACCCGAC CCACCACCGC CCGATCCACC GCCTCCTGAG GAGACGGTGA CCGTGGTC

58

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGTGGATCGG GCGGTGGTGG GTCGGGTGGC GCGGGATCTG ACATCCAGCT GACCCAGAGC

60

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TGCAGGATCC AACTGAGGAA GCAAAGTTTA AATTCTACTC ACCTTTGATC

50

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

28

(ii) MOLECULE TYPE: DNA (genomic)

48

(ii) MOLECULE TYPE: DNA (genomic)

27

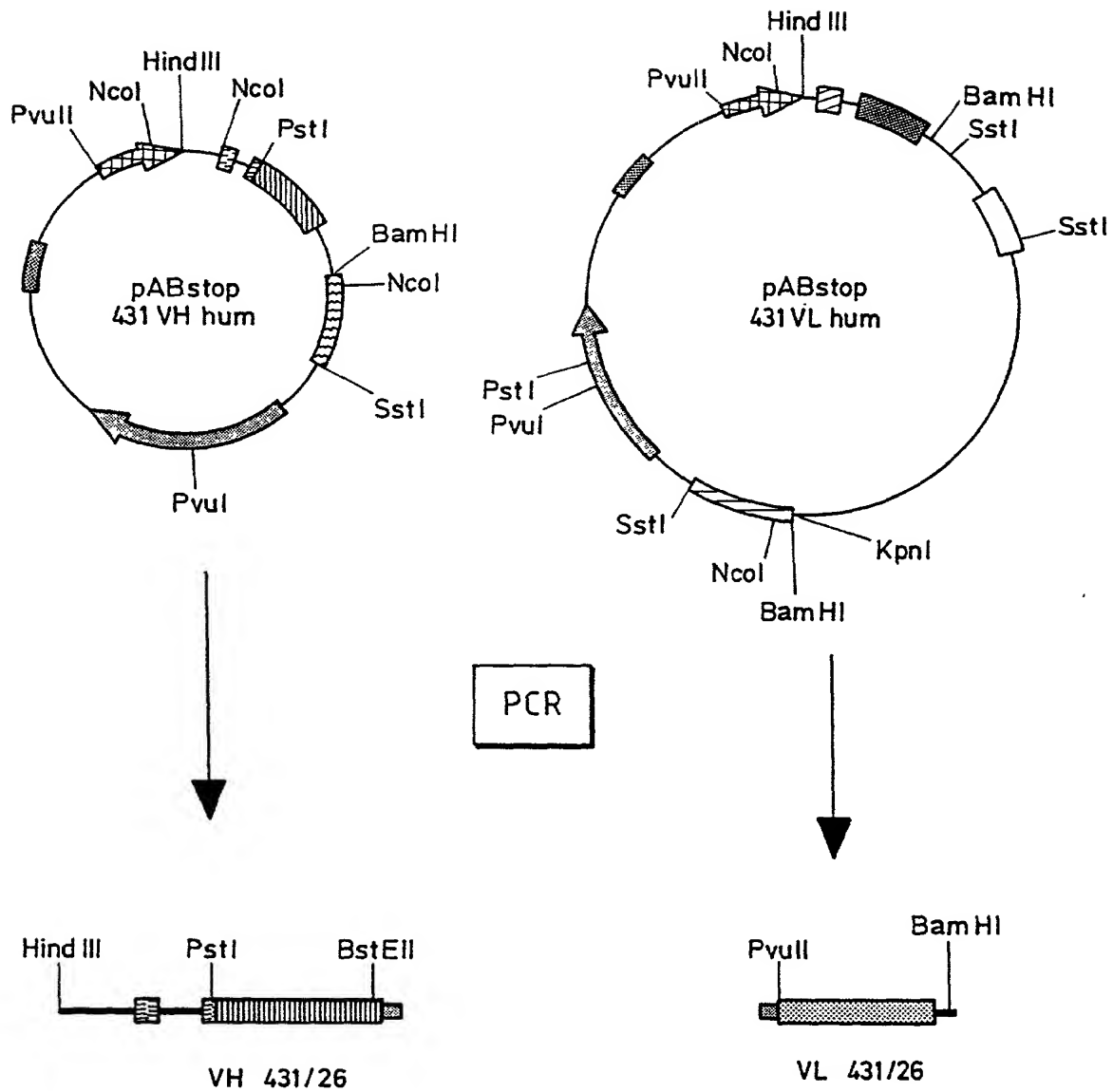


FIG.1

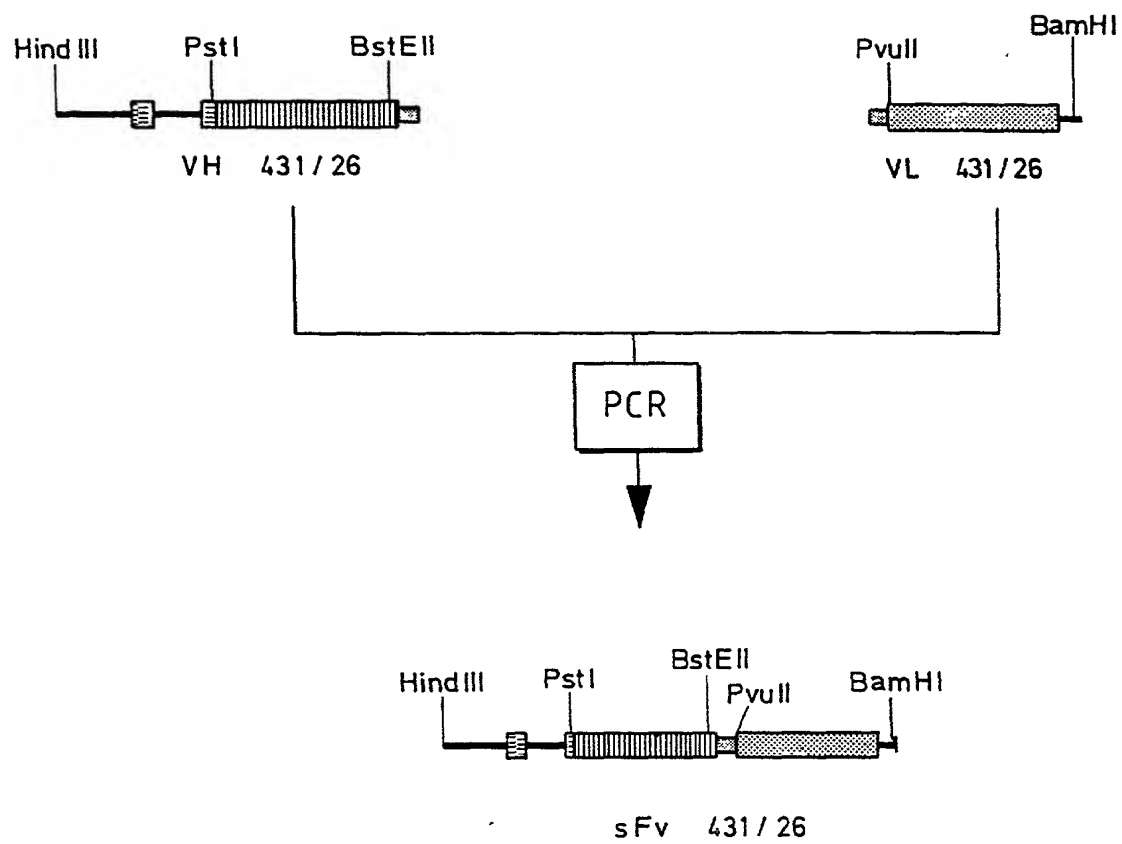


FIG.2

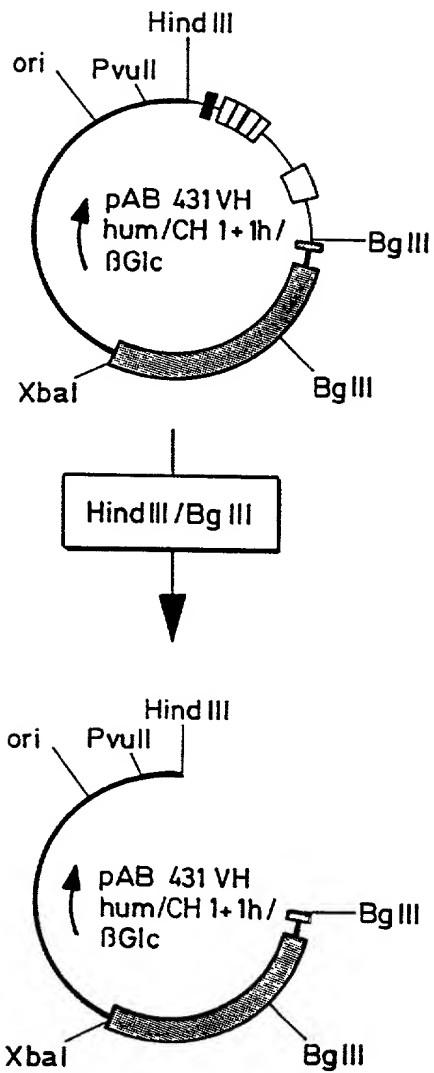
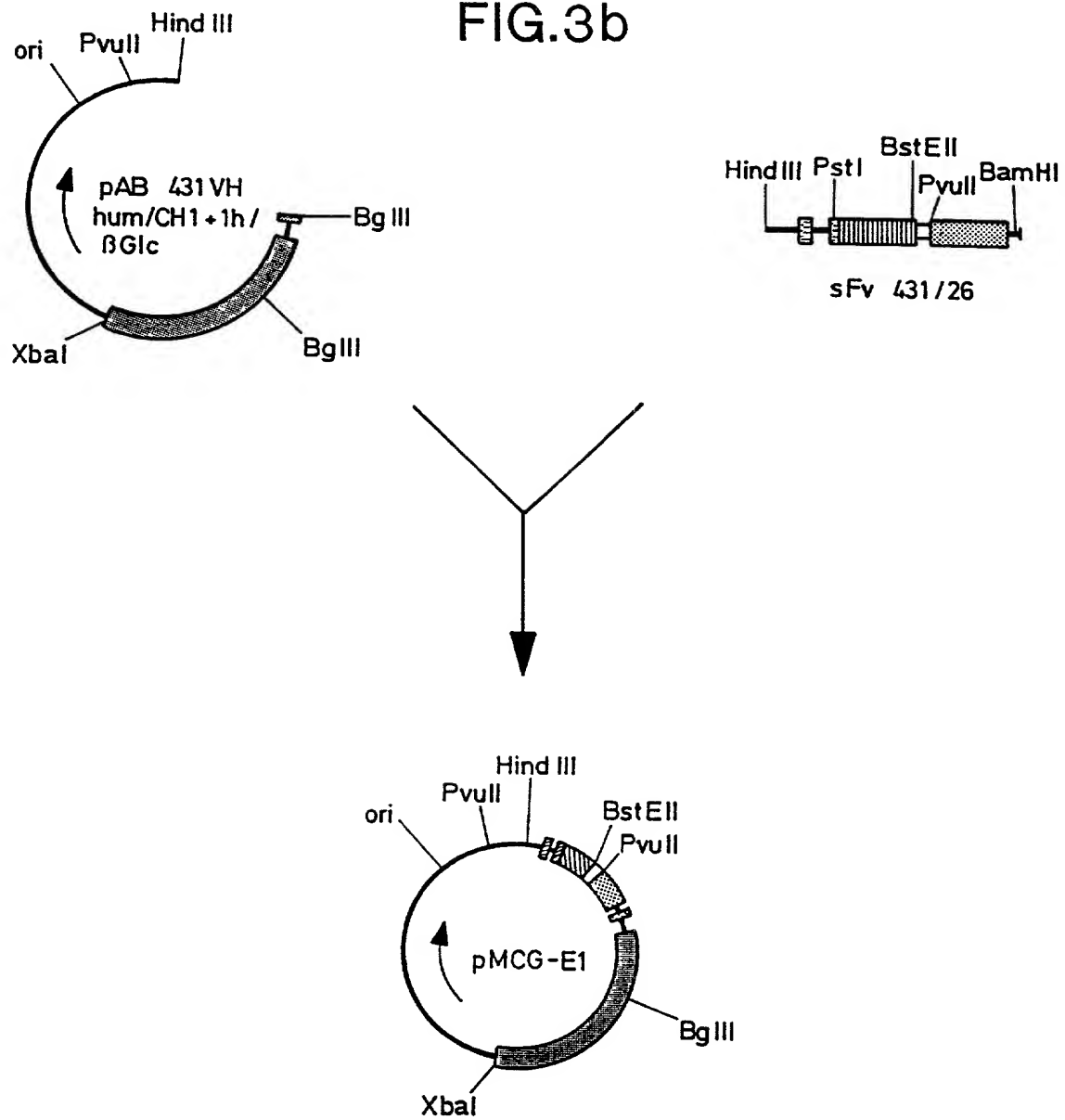
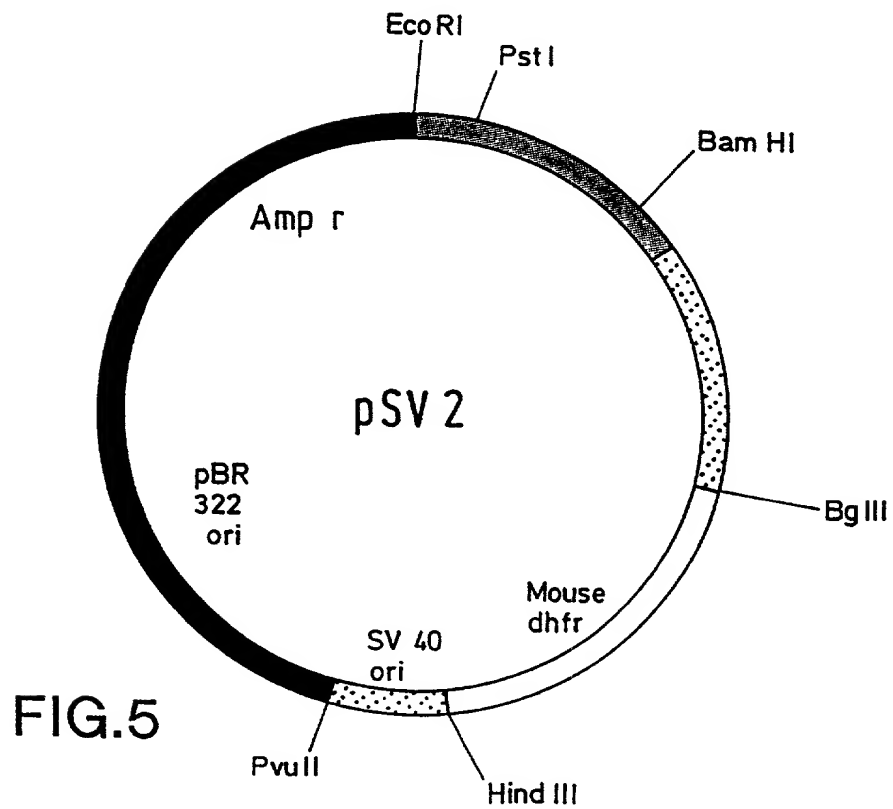
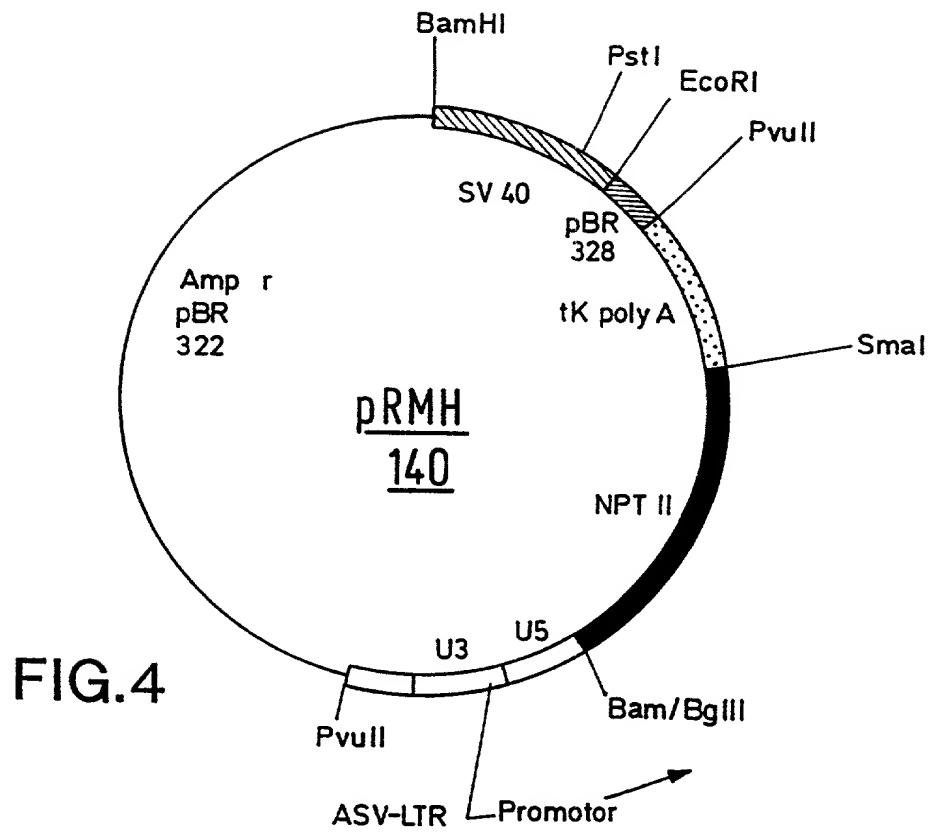
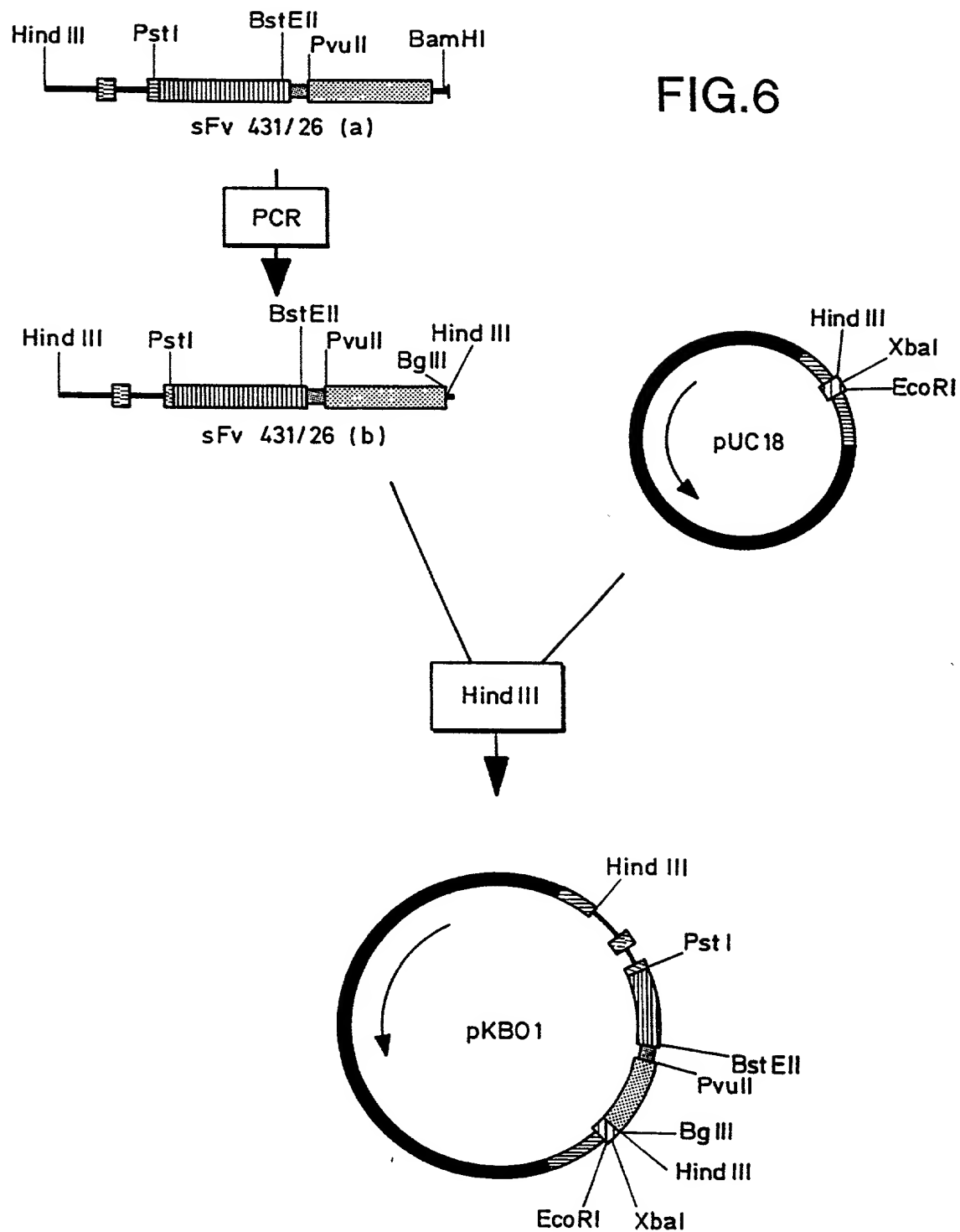


FIG.3a

FIG.3b







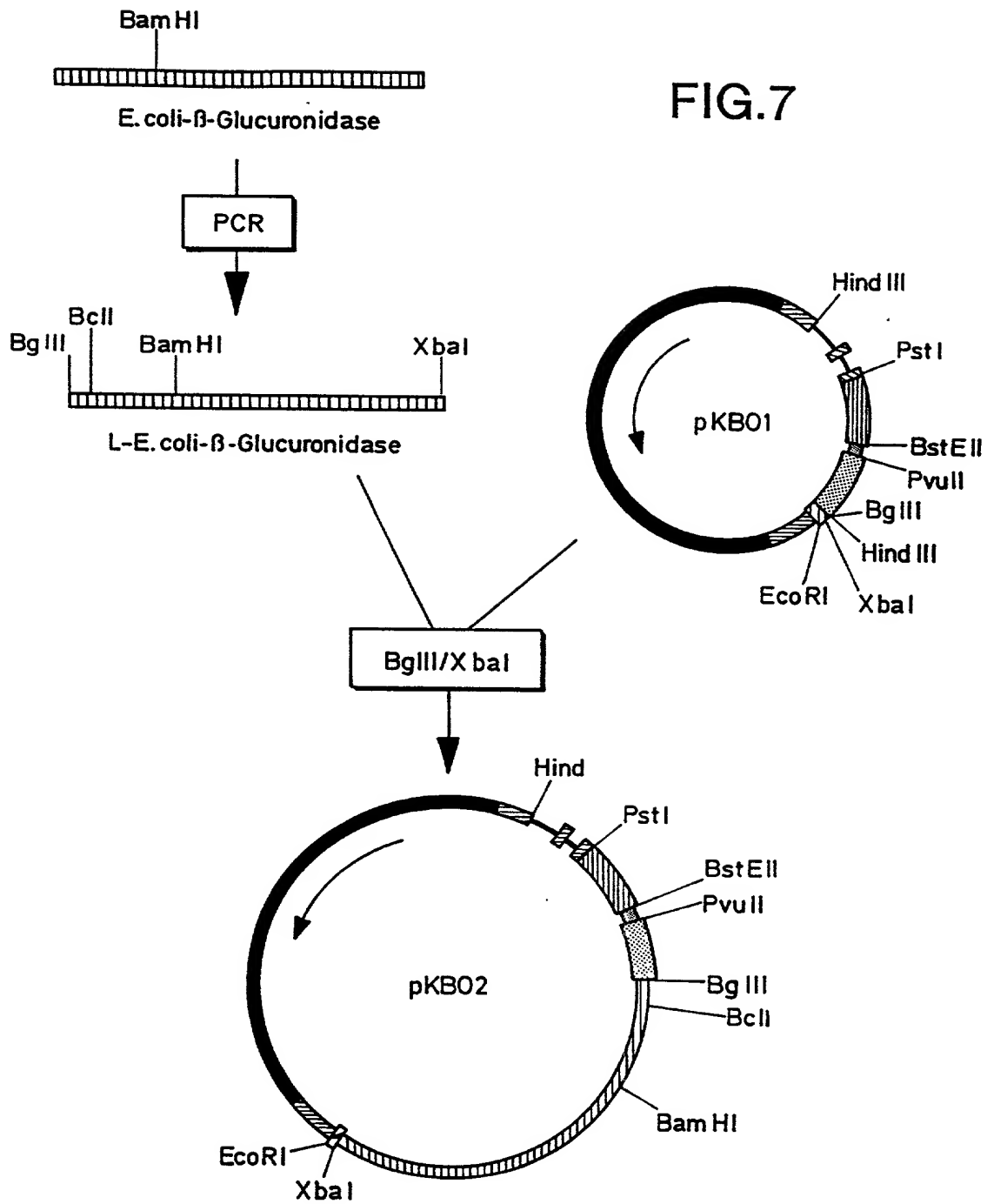
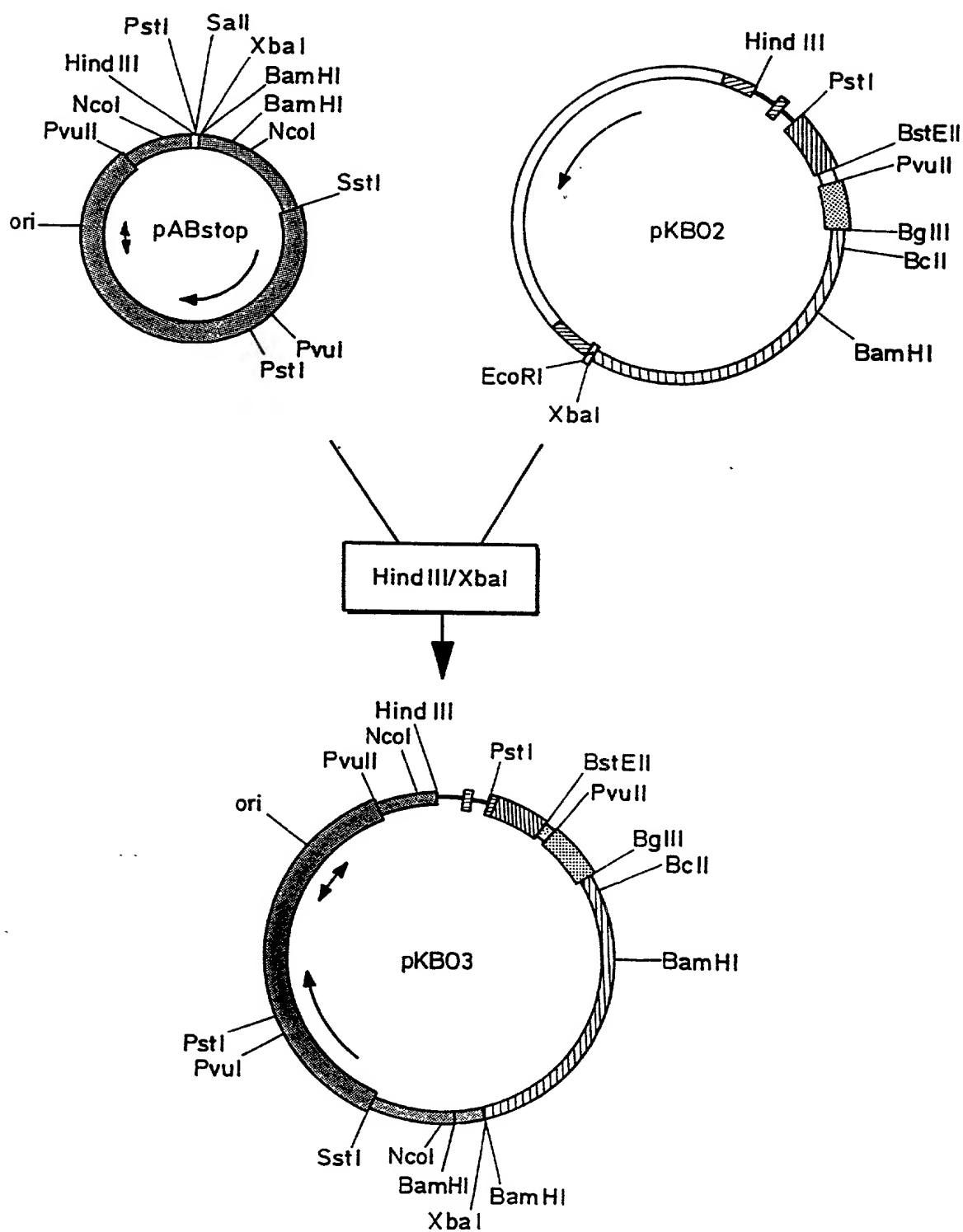


FIG.8



DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

BIFUNCTIONAL GLYCOPROTEINS HAVING A MODIFIED CARBOHYDRATE COMPLEMENT, AND THEIR USE IN TUMOR-SELECTIVE THERAPY

the specification of which (check one)

☐ is attached hereto

☒ was filed on April 29, 1994 as Application Serial No. 08/235,395 and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is known by me to be material to patentability as defined in Title 37, Code of Federal Regulations § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

PRIOR FOREIGN APPLICATION(S)

NUMBER	COUNTRY	DAY/MONTH/YEAR FILED	PRIORITY CLAIMED
<i>P 43 14 556.6</i>	<i>Germany</i>	<i>04/MAY/1993</i>	<i>Yes</i>
<i>94106394.3</i>	<i>European</i>	<i>25/APRIL/1994</i>	<i>Yes</i>

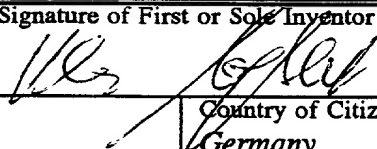
I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is known by me to be material to patentability as defined in Title 37, Code of Federal Regulations § 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION SERIAL NO.	FILING DATE	STATUS: PATENTED, PENDING, ABANDONED

I hereby appoint as my attorneys, with full powers of substitution and revocation, to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: Stephen A. Bent, Reg. No. 29,768; David A. Blumenthal, Reg. No. 26,257; John J. Feldhaus, Reg. No. 28,822; Donald D. Jeffery, Reg. No. 19,980; Peter G. Mack, Reg. No. 26,001; Brian J. McNamara, Reg. No. 32,789; Sybil Meloy, Reg. No. 22,749; George E. Quillin, Reg. No. 32,792; Colin G. Sandercock, Reg. No. 31,298; Bernhard D. Saxe, Reg. No. 28,665; Richard L. Schwaab, Reg. No. 25,479; Arthur Schwartz, Reg. No. 22,115.

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Address telephone communications to Colin G. Sandercock at (202) 672-5300.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full Name of First or Sole Inventor Klaus BOSSLET	Signature of First or Sole Inventor 	Date <i>June 1, 94</i>
Residence Address Marburg, Germany	Country of Citizenship Germany	
Post Office Address An der Haustatt 64, D-35037 Marburg, Germany		

Signatures should conform to names as typewritten. ☒ Additional inventors on attached Page 2.

